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(54) **Stable formulation of biologically active proteins for parenteral injection.**

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Description

This invention is in the field of biochemical engineering. More particularly, the invention concerns a pharmaceutical formulation of biologically active recombinant IL-2 or IFN- β protein which is suitable for parenteral injection into humans.

Naturally occurring interferons (IFNs) are species-specific proteins, often glycoproteins, produced by various cells upon induction with viruses, double stranded RNA's, other polynucleotides, antigens and mitogens. Interferons exhibit multiple biological activities such as antiviral, antiproliferative, immunomodulatory and anticellular functions. At least three distinct types of human interferons have been identified and characterized in terms of their anti-viral, anti-growth and activation of natural killer cell (NK) activities. They are produced by leukocytes, lymphocytes, fibroblasts and the immune system and are classified α , β and γ interferons. These are reported to be different proteins coded for by distinct structural genes.

In recent times, however, several of the human interferon genes have been cloned using recombinant DNA technology and expressed in *E. coli* (Nagola, S. et al., *Nature*, 284:316 (1980); Goeddel, D. V. et al., *Nature*, 287:411 (1980); Yelverton, E. et al., *Nuc Acid Res.*, 9:731 (1981); and Streuli, M. et al., *Proc Natl Acad Sci (USA)*, 78:2848 (1981).

Native human IL-2 is an antigen-nonspecific, genetically unrestricted soluble factor produced by erythrocyte rosette positive T cells stimulated with antigens, mitogens and alloantigens. It is a protein with a reported molecular weight in the approximate range of 13,000 to 17,000 daltons (S. Gillis and J. Watson, *J Exp Med* (1980) 159:1709) and an isoelectric point in the approximate range of pH 6-8.5. Human IL-2 has a number of *in vitro* and *in vivo* effects including enhancing the proliferative responses of human peripheral blood mononuclear cells or murine thymocytes, enhancing the immune response in humans and in animals against bacterial, parasitic, fungal, protozoan and viral infections, and supporting the growth of continuous T cell lines.

Proteins such as IFNs and IL-2 and muteins thereof in which a cysteine residue is deleted (in the case of IFN) or replaced with another amino acid (in the case of IFN and IL-2) have been produced microbially through genetic engineering techniques. Microbially produced IL-2 is not glycosylated and is produced in a reduced state by the microorganisms. When purified and oxidized, these microbially produced IL-2s exhibit activity comparable to native human IL-2.

Procedures for purifying native IL-2 from T cells are described by Watson, J., et al, *J Exp Med* (1979) 150:849-851; Gillis, S., et al, *J Immunology* (1980) 124:1954-1962; Mochizuki, D. Y., et al, *J Immun Meth* (1980) 39:185-201; Welte, J., et al, *J Exp Med* (1982) 156:454-464; and European Patent Applications 83103582.9 (published October 26, 1983 under no. 92163) and 83403582.9 (published November 16, 1983 under no. 94317). In general, these procedures involve precipitating proteins from culture supernatants with ammonium sulfate followed by a chromatographic fractionation.

Procedures for recovering and purifying bacterially produced IFNs are described in U.S. Patent Nos. 4,450,103; 4,315,852; 4,343,735; and 4,343,736; and Derynck, R., et al, *Nature* (1980) 287:193-197 and Scandella and Kornberg, *Biochemistry*, 10:4447 (1971). Generally with these methods the IFN is not produced in a sufficiently pure form and in sufficiently large quantities for clinical and therapeutic purposes and the resulting IFN preparations produced by recombinant DNA techniques have residual toxic amounts of chemicals, such as sodium dodecyl sulfate (SDS) and other surfactants or precipitants used in the extraction and purification steps which are not acceptable for clinical studies in therapeutic applications.

Both recombinant IFN- β and IL-2 are insoluble in solutions which are at a physiological pH of 7 to 8. Therefore, various processes and additives have been devised to solubilize these proteins.

U.S. Patent No. 4,462,940 to Hanisch et al. discloses a process for formulating interferon by mixing the interferon and normal serum albumin at pH 12.0 for 5 minutes and then adjusting the pH to 7.5 to obtain a soluble mixture. Exposing the interferon to such a high pH may adversely affect the properties of the interferon. In addition, the formulation contains some SDS.

The only alternatives to the high pH albumin formulation are the addition of detergents or of chaotropic agents such as urea or guanidine hydrochloride to the formulation to solubilize the protein. A detergent such as sodium dodecyl sulfate will cause hemolysis upon injection of its concentration is sufficiently high. Additionally, even a relatively low level of SDS where approximately one molecule of SDS is bound to each molecule of IL-2 may cause liver damage, if the IL-2 formulation is administered at very high doses. Chaotropic agents such as urea work to solubilize IL-2 but must be used at very high concentrations of 30% or higher solutions. In addition, with urea there are problems with chemical modification of the protein at high concentrations.

A reagent-grade IL-2 which is in solution with the detergent N-lauryl sarcosinate has been commercialized. In addition, an IL-2 formulation is being sold which contains reagent-grade IL-2 and is recom-

mended to be reconstituted in a complete tissue culture medium with 0.5% deoxycholate, another detergent. Such formulations, however, are not suitable for parenteral injection into humans.

Fatty acids have been used as pharmaceutical absorption promoters for oral and vaginal administration and for rectal application. See, e.g., Jap. Kokai 8288126 and 8280314 to Kyoto Pharmaceutical Industries. See also Jap. Kokai 81104812 to Sankyo Co., Ltd. In addition, fatty acids are known for use in topical creams (U.S. Patent No. 4,331,653), in effecting dissolution of solutes in water (U.S. Patent No. 3,667,929) and for pharmaceutical injection (U.S. Patent No. 3,658,970-lauric acid).

Wang et al., *J Parenteral Drug Assoc*, 34, 452-462 (1980) provides a review of excipients and pH's for parenteral products used in the United States. This article indicates that it is not trivial to choose excipients for parenteral products as for other dosage forms due to concerns for safety and feasibility in sterilization. Acceptance of a substance to be used as excipient often involves lengthy safety testing or production trials. Also, for reasons of stability or solubility the pH of the product cannot always be adjusted to physiological pH of 7.4. A list of solubilizing agents such as detergents and lipids in use for various drugs is provided on p. 454 and a list of stabilizers, including sodium caprylate (octanoate) for normal serum albumin, is provided on p. 458.

There is a need in the art for a protein formulation where the protein is not subjected to high alkalinity so as to alter the protein, the protein is soluble and the formulation is free or virtually free of SDS.

The present invention provides a stable pharmaceutical composition of matter suitable for parenteral injection into animals or humans comprising a therapeutically effective amount of a recombinant interleukin-2 or β -interferon protein purified to contain less than 4 μ g sodium dodecyl sulfate per mg protein dissolved in an inert carrier medium comprising sodium laurate at a pH of 7.0 to 8.0. In such a formulation the IL-2 is in a soluble form at physiological pH and the pH need not be raised to above 10.5 to obtain such a soluble formulation. Moreover, sodium laurate naturally occurs in the blood. A compound similar to sodium laurate, sodium octanoate, which is a known stabilizer for human serum albumin, is not an effective stabilizer for the biologically active protein IL-2.

Thus, in general the invention provides the use of sodium laurate in stabilizing an interleukin-2 interferon- β so as to render said protein suitable for administration to animals or humans.

This composition is prepared by an improved process comprising (a) extracting the recombinant protein (IL-2 or IFN- β) from a disruptate of a host organism transformed to produce the protein, (b) purifying the extracted protein to contain less than 4 μ g sodium dodecyl sulfate per mg protein, (c) mixing the purified protein with an inert carrier medium at a pH of about 9 to 9.5 in the presence of sodium laurate, and (d) adjusting the pH of the mixture to between 7.0 and 8.0.

In preferred embodiments of this invention, the stabilizer is present in a concentration of 0.03 to 0.1% by weight, depending on the exact pH of the solution, the solubilizing agent used in the process is SDS, the formulation further comprises mannitol, the pH thereof is 7.5 to 7.7, and the formulation is lyophilized after step (d).

Figure 1 shows a flow diagram of a preferred procedure for processing microbially produced IL-2 and formulating it at pH 8.0.

Figure 2 shows a flow diagram as in Figure 1 except that the formulation is adjusted to pH <8.0.

The term "recombinant" refers to IL-2 and IFN- β proteins generated using genetic engineering techniques. These techniques typically involve identifying and characterizing the structural gene which encodes the native protein, isolating or synthesizing that gene or a mutant which encodes a functionally equivalent mutein of the native protein, inserting the gene into an appropriate expression vector in a position which permits expression of the gene, transforming competent hosts with the vector, identifying correct transformants, and culturing the transformants in a suitable growth medium. The host organism may be yeast or mammalian cells, but is preferably microorganisms, more preferably bacteria, and most preferably *E. coli*. The IL-2 and IFN- β are typically recovered from the culture by disrupting the cells, treating the cellular debris with solubilizing agents (depending on the solubility characteristics of the protein) and one or more extractants to isolate the crude protein, and purifying the crude protein by various preparative chromatographic procedures. If the protein is susceptible to oligomer formation during the fermentation or recovery processes, the protein may be treated with a reducing agent at an appropriate stage in the recovery process.

As used herein the term " β -HIFN" refers to human β -interferon or β -interferon-like polypeptides produced by recombinant DNA techniques, and whose amino acid sequence is the same, similar or substantially homologous to native β -interferon, both the glycosylated and the unglycosylated protein. Examples of such recombinant proteins are the IFNs described by Nagola, S. et al., *Nature*, 284:316 (1980), Goeddel, D. V. et al., *Nature*, 287:411 (1980), Yelverton, E. et al., *Nuc Acid Res*, 9:731 (1981), Streuli, M. et al., *Proc Nat'l Acad Sci (USA)*, 78:2848 (1981), European Patent Publication No. 28,033 published June 6,

1981 and European Patent Application No. 109,748 published May 30, 1984.

As used herein the term "IL-2" denotes a protein which is native interleukin-2 or is produced by a host that has been transformed with a human interleukin-2 gene or a modification of the human interleukin-2 gene that encodes a protein having: (a) an amino acid sequence that is at least substantially identical to the amino acid sequence of native human interleukin-2 and (b) biological activity that is common to native human interleukin-2. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (additions, substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and native human interleukin-2. Examples of such recombinant proteins are the IL-2s described in European Patent Application 83101035.0 filed February 3, 1983 (published October 19, 1983 under publication no. 91539), European Patent Application 82307036.2 filed December 22, 1982 (published September 14, 1983 under no. 88195), European Patent Application 83306221.9 filed October 13, 1983 (published May 30, 1984 under no. 109748), and the IL-2s described in the examples of this application.

The IL-2 and β -HIFN particularly preferred herein are muteins of biologically active IL-2 and β -HIFN in which one or more cysteine residues which are not essential to biological activity have been deliberately deleted or replaced (in the case of β -HIFN) or replaced (in the case of IL-2) with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation. More preferably the protein herein is a IFN- ρ mutein, with the cysteine residue at amino acid position 17 of the native counterpart replaced by a serine residue (designated IFN- β_{Ser17}) or an IL-2 mutein with the cysteine residue at amino acid position 125 of the native counterpart replaced by a serine residue (designated IL-2 $_{\text{Ser125}}$), or with the initial alanine residue of the native counterpart eliminated, or with the initial alanine residue eliminated and the cysteine at position 125 replaced by serine (designated des-Ala-Ser $_{125}$ IL-2).

The composition herein is formulated in a medium defined further below containing an effective amount of a particular type of stabilizer which is designed to dissolve the protein in the medium and which is non-toxic and therapeutically compatible.

The word "inert" in describing the medium refers to media which in the formulation will not interfere with the biological activity of the IL-2 or β -HIFN protein contained in the formulation.

In general, the recovery, purification and formulation process herein involves fermenting the host organism transformed to express the protein, disrupting the cell membrane of the host organism, extracting the bacterial proteins from the disruptate using low concentrations of a chaotropic agent, recovering the protein using a solubilizing agent, extracting the protein with 2-butanol or 2-methyl-2-butanol, subjecting the extracted protein to chromatographic purification, precipitating the protein by neutralization, redissolving the precipitate in a chaotropic agent, removing the SDS, formulating the protein at pH 9 to 9.5 in the medium with the sodium laurate, adjusting the pH of the formulation to between 7.0 and 8.0, inclusive, and lyophilizing the formulation.

The preferred embodiment of the process of this invention for formulating recombinant IL-2 produced from microorganism hosts is summarized below.

The transformed microorganisms may be grown in a suitable growth medium, typically to an optical density (OD) of at least about 30 to 680 nm, and preferably between about 20 and 40 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The medium is an aqueous medium containing compounds that fulfill the nutritional requirements of the microorganism. Growth media will typically contain assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (See Review of Medical Biology, Lange Medical Publications, 14th Ed pp 80-85 (1980).) In expression vectors involving the trp promoter, the tryptophan concentration in the medium is carefully controlled to become limiting at the time IL-2 expression is desired. Growth media for E. coli are well known in the art.

After the cells are harvested from the culture, they may be concentrated, if necessary, to about 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm) by filtration, centrifugation, or other conventional methods.

Following concentration the cell membranes of the transformed microorganisms may be disrupted. The main purpose of disruption is to facilitate the following extraction and solubilization steps. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling may be used in this step of the process. Preferred methods are sonication or homogenization with a Manton-Gaulin homogenizer. The end point of the disruption step may be monitored by optical density, with the optical density of the suspension typically decreasing about 65% to 85%. In any event, the disruption should break substantially all of the cells so that substantially no intact cells are carried through to the solubilization step. Before the disruption, the pH of the liquid phase of the concentrate is adjusted, if necessary, to a level that facilitates removal of E. coli proteins in subsequent steps, while retaining IL-2 protein as an insoluble complex in the

cellular debris. The pH may be so adjusted by adding suitable buffers. In most instances pHs in the range of 8 to 8.5 will be used.

The steps in the recovery process subsequent to the disruption step are primarily designed to separate the IL-2 from *E. coli* proteins to a high level of purity (preferably at least about 95% and more preferably at least about 98%) in good yields while maintaining the IL-2 in a reduced state. Simultaneously, these purification processes, in combination, also reduce pyrogenic substances in the final product to a level believed to be acceptable for parenteral administration to patients.

After the cells have been disrupted the particulate matter may be separated from the liquid phase of the disruptate and resuspended in an aqueous medium buffered to the optimal pH for the extraction. The particulate matter may optionally be washed with buffer at this stage to remove any water soluble *E. coli* proteins therein. In any event, the protein concentration of the cell suspension subjected to the extraction will usually be in the range of 5 to 60 mg/ml, preferably 20 to 40 mg/ml.

The extraction of *E. coli* proteins from the particulate cellular material may be carried out concurrently with the disruption or sequentially following the disruption. It is preferably carried out as a separate step following the disruption. The extractant is an aqueous solution of a chaotropic agent (i.e., a mild protein denaturant that dissociates hydrogen bonds and affects the tertiary structure of proteins). The extractant selectively removes the bulk of the *E. coli* proteins from the cellular debris leaving at least a substantial portion of the IL-2 associated (contained in or bound to) with the cellular debris. The selectivity is facilitated by the hydrophobicity of the IL-2 and the fact that it is in a reduced, insoluble state at a pH near the isoelectric point of the protein. In addition, a substantial portion of the IL-2 may be present in vivo as inclusion bodies of significant mass, as has been the case with other cloned proteins expressed at high levels in *E. coli*. Examples of extractants include sodium laurate, urea, guanidinium hydrochloride (guanidinium hydrochloride should not be used when SDS is used as a solubilizing agent), and sodium thiocyanate. Urea is preferred. The concentration of the chaotropic agent in the extraction mixture will depend upon the particular agent that is used and the amount of cellular material in the extraction mixture. In the case of urea, concentrations (final) between about 3.5 M and 4.5 M, preferably about 4 M, will be used in batch processes at 25°C. If the extraction is run on a continuous basis over longer time periods it may be desirable to use lower concentrations. Temperatures in the range of 20°C to 25°C will normally be used in extraction, with room temperature being used for convenience. Mixing will typically be used to enhance contact between the solution and particulate matter and thus decrease the time required to extract non-IL-2 proteins from the cellular debris. Kinetic analysis of the extraction process was performed on the supernatants using SDS-PAGE, and the extraction was found to be essentially complete by 15-30 minutes.

Following the extraction, the mixture may be separated into solid and liquid phases. The IL-2 in the solid phase is then selectively solubilized by contacting the solid phase with a neutral, aqueous buffer containing a reducing agent and a solubilizing agent. Surface active agents (detergents) that have a suitable hydrophobic-hydrophilic balance to solubilize the hydrophobic IL-2 may be used. Alkali metal sulfates containing 10 to 14 carbon atoms and alkali metal alkyl sarcosinates are preferred solubilizing agents, with SDS and sarcosyl being particularly preferred.

The amount of solubilizing agent used in the solubilization will depend upon the particular agent. When SDS or sarcosyl are used, the preferred ratio (w/w) of SDS/sarcosyl to solid phase protein is about 0.5:1 to 1.4:1. The solubilizing medium also contains a sufficient amount of reducing agent to prevent the solubilized IL-2 from undergoing oxidation to any significant degree. Protein reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol may be used. The concentration of reducing agent such as DTT in the medium will usually range between 5 to 20 mM. The solubilization will typically be carried out at temperatures in the range of 20°C to 25°C with mixing to facilitate contact between the solid phase and the solubilizing medium. Higher temperatures may solubilize unwanted *E. coli* proteins. The solubilization is considered complete when the sample has sat 15 minutes or the solution turns translucent. Insoluble material is separated after completing the solubilization.

After the IL-2 is solubilized the IL-2 may be extracted from the aqueous solution under reducing conditions with 2-butanol or 2-methyl-2-butanol to remove additional *E. coli* proteins, notably including certain contaminants that have molecular weights very close to the IL-2. Conditions (e.g., ionic strengths in the range of 0.05 and 0.15) at which the aqueous solution and butanol are substantially immiscible are used. In carrying out the organic extraction the protein concentration of the aqueous solution is preferably adjusted, if necessary, to less than about 6 mg/ml, preferably 0.5 to 4 mg/ml. Reducing conditions are maintained by carrying out the extraction in the presence of a reducing agent (e.g., DTT). The butanol will normally be added to the aqueous solution of solubilized IL-2 in volume ratios in the range of 1:1 to 3:1 (extractant:aqueous solution), preferably about 1:1. The extraction may be carried out in a batch or continuous operation. The temperature will normally be in the range of 20°C to 100°C and the pH will

normally be 4 to 9, preferably 5 to 6. The time of contact between the solution and the butanol is not critical and relatively short times on the order of a few minutes may be used. After the extraction is complete, the aqueous phase and butanol phase are separated and the IL-2 is separated from the butanol phase. A preferred procedure for separating the IL-2 from the butanol phase is acid precipitation. This is done by adding the butanol phase to aqueous buffer, pH 7.5, until the organic phase is dissolved (approximately 2-3 volume buffer per volume of organic), and then lowering the pH to 5.5 to 7.0, preferably 6.0 to 6.2, to cause the IL-2 to precipitate.

In a subsequent step the IL-2 may be separated from any *E. coli* contaminants remaining after the extraction(s) and optimally from the solubilizing agent. Gel filtration chromatography, RP-HPLC, or a combination of gel filtration chromatography and RP-HPLC is used. The gel filtration chromatography is preferably carried out in two stages that remove both pyrogenic components and protein contaminants having molecular weights higher or lower than IL-2. (IL-2 has a molecular weight of about 15.5K daltons.) Gels that are capable of fractionating the solution to permit separation of the IL-2 from these contaminants are commercially available. Sephacryl S-200 is a preferred gel for removing the higher molecular weight components and Sephadex G-25, G-75 or G-100 gels are preferred for removing the low molecular weight contaminants. The gel filtrations will typically be run in buffered solutions (pH 5.5 to 7.0) containing about 0.1% to 1.0% solubilizing agent and about 1 to 10 mM reducing agent. The column will be sized to permit suitable resolution of the desired components.

RP-HPLC is an alternative to gel filtration. Also, RP-HPLC is capable of removing molecules from the solution that have molecular weights close to IL-2 and cannot, therefore, be removed completely by gel filtration. In addition, contaminants such as bacterial endotoxin are also removed effectively by RP-HPLC. Therefore, RP-HPLC may also be used as a final purification step after gel filtration. Supports (stationary phases) that provide good resolution of proteins may be used in the RP-HPLC. C-4, C-8, or C-18 on 300 angstrom pore-size supports are examples of preferred supports. The separation is carried out at an acidic pH of less than 2.3, usually 2.1 to 2.3, in order to keep the IL-2 in solution. In this regard, the pH of the solution from the solubilization (gel filtration) will preferably be adjusted to this range. The solution is loaded into the RP-HPLC column and is adsorbed onto the stationary phase. A gradient solvent system comprising an organic acid such as acetic acid or trifluoroacetic acid and organic solvent such as propanol or acetonitrile may be used to elute the IL-2 from the column. Acetic acid-propanol, trifluoroacetic acid-propanol, and trifluoroacetic acid-acetonitrile are preferred solvent systems. IL-2 elutes in the acetic acid-propanol system at about 40% propanol, in the trifluoroacetic acid-propanol at about 50% propanol, and in the trifluoroacetic acid-acetonitrile system at about 62% acetonitrile. For convenience, the organic solvent content of the elutant will usually be increased rapidly to a level somewhat below the solvent concentration at which the IL-2 elutes followed by a slow gradient change in the range of about 0.1% to 1.0%/min. The most preferred solvent system is trifluoroacetic acid-acetonitrile, especially 0.1% trifluoroacetic acid-acetonitrile, because it is a higher resolution system than the acetic acid-propanol system.

As soon as the IL-2 is recovered from the chromatography step it is precipitated by neutralization with a base such as Na_2HPO_4 or NaOH and redissolved in a chaotropic agent as defined above, subjected to gel filtration desalting column such as a G25 Sephadex column. The chaotropic agent may be, for example, sodium laurate, guanidine hydrochloride, urea or sodium thiocyanate. Preferably it is sodium laurate or urea. Generally the redissolving takes place in the presence of a neutralizing base depending on the solvent system used for the previous chromatography step.

After desalting, the IL-2 is collected and combined with an effective amount of sodium laurate to dissolve the IL-2. The exact amount of sodium laurate employed will depend mainly on the ultimate pH of the formulation, as well as the concentration of IL-2. Generally, an effective amount at about pH 7.5 is from 0.01% to 0.1% by weight per volume, preferably 0.03 to 0.1% by weight per volume. Below 0.01% the sodium laurate is not effective as a solubilizing agent; above 0.1% the IL-2 will precipitate from the solution. At a higher pH these optimum concentrations may vary.

The initial mixing of IL-2 and sodium laurate in water will take place at pH 9-9.5. After the ingredients are mixed the pH is adjusted downward to 7.0 to 7.8 inclusive, and the components are again mixed. The specific pH is critical because at certain pH values within the range of 7.0 to 8.0 the protein will not be soluble or the sodium laurate will precipitate or otherwise be ineffective. For example, if the pH is adjusted to below 7.4-7.5 the sodium laurate will precipitate as lauric acid. A final pH of 7.5 to 7.7 is therefore preferred.

The reagents used to adjust the pH upward or downward are not critical. Thus, any base, preferably an inorganic base such as dibasic sodium phosphate (Na_2HPO_4), sodium hydroxide, or ammonium hydroxide, may be used to adjust the pH to 9-9.5, and any acid, such as HCl, maleic acid, lactic acid, tartaric acid or citric acid, may be used to adjust the pH downward. The acid is preferably used in concentrated form so

that the amount used will not effectively dilute the mixture so as to precipitate the IL-2.

For parenteral administration the IL-2 will be formulated in a unit dosage injectable solution form in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin in water. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as bulking substances or toxicity modifiers such as glycerine, lactose, mannitol or dextrose, oleaginous vehicles such as benzyl benzoate, lubricants, suspending agents, chelating agents, stabilizers, or substances that enhance isotonicity and chemical stability, e.g., buffers such as tartaric acid, antimicrobial preservatives such as phenol, and antioxidants such as acetone sodium bisulfite. The IL-2 will typically be formulated in such vehicles at concentrations of 0.1 mg/ml to 10 mg/ml, preferably 0.2 to 5 mg/ml. A preferred additive for the IL-2 formulation is mannitol, in a concentration of 2.5 to 5.0% by weight of the formulation.

After the formulation has been adjusted to a pH of 7.0 to 8.0, the formulation is preferably filtered to obtain a clear solution if the pH is adjusted to less than 8.0 as opposed to exactly 8.0, lyophilized, and finally resuspended in a neutral aqueous buffer containing the reducing agent (if necessary to keep the IL-2 in a reduced state) and the sodium laurate (to keep it in solution). The IL-2 is stable in this form and may be stored for further treatment and formulation before being used.

Figure 1 illustrates the details of the individual process steps of the present invention including the culture of the transformed microorganisms in an appropriate fermentation medium through the final step where the purified IL-2 is lyophilized for reconstitution into therapeutic formulations. In this scheme a further step involves oxidation of IL-2 after it has been separated by gel filtration and purification of the oxidized product by RP-HPLC or gel filtration followed by RP-HPLC. This results in efficient removal of contaminants surviving the gel filtration as well as unwanted oxidation products. A preferred oxidation procedure is to oxidize a fully reduced microbially produced synthetic IL-2 having an amino acid sequence substantially identical to a useful IL-2, which sequence includes cysteines which in the useful IL-2 are linked intramolecularly to form a cystine in a controlled manner so that the cysteines are oxidized selectively to form the cystine. In this process the fully reduced microbially produced synthetic IL-2 is reacted with an oxidizing agent that oxidizes cysteines selectively in an aqueous medium at a pH at least about one-half pH unit below the pK_a of said cysteines. The concentration of synthetic protein in this reaction mixture is less than about 5 mg/ml and the mol ratio of oxidizing agent to protein is at least stoichiometric, with the proviso that the oxidizing agent is in excess in the terminal portion of the reaction. One preferred oxidizing agent is o-iodosobenzoic acid. RP-HPLC purification of the oxidized product may be carried out under the conditions described above in the absence of a reducing agent and in the presence of a detergent at a concentration equal to or less than those used in the above described gel filtration.

The purity of the IL-2 after the chromatography step(s) is at least about 95% and usually at least about 98%. This highly pure material contains less than about 5 ng endotoxin, usually less than about 0.01 ng endotoxin per 1,000,000 International Units IL-2 activity.

The HPLC pool of oxidized IL-2 is precipitated by adding Na_2HPO_4 . The precipitate is then collected by centrifugation and redissolved in sodium laurate and Na_2HPO_4 . The redissolved precipitate is loaded on a gel filtration column, such as a Sephadex G-25 column, and the IL-2 is collected from the column. The IL-2 collected is virtually free of solubilizing agent and sodium laurate. This collected IL-2 is then formulated with the sodium laurate at pH 9.1 and then the pH is adjusted to 8.0 using 1 M HCl.

The formulation is then lyophilized and ready for use. This process results in a formulation which is virtually free of SDS, yet is in solution form.

Figure 2 illustrates the embodiment where the formulation of sodium laurate and IL-2 at pH 9.1 is adjusted to a pH less than 8.0 with 1 M HCl. In this instance, the adjusted composition is preferably filtered to remove excess sodium laurate to obtain a clear solution and is then lyophilized.

The IL-2 composition herein may be used to enhance the proliferative responses of human peripheral blood mononuclear cells or murine thymocytes, to enhance the immune response in humans and in animals against bacterial, parasitic, fungal, protozoan and viral infections, and to support the growth of continuous T cell lines.

In a preferred embodiment for formulating β -HIFN, the individual process steps are summarized below:

- growing the transformed bacterial hosts in an appropriate fermentation medium;
- concentrating the bacteria in the fermentation medium by cross-flow filtration;
- mechanically homogenizing the bacteria to disrupt the cell walls of the bacteria;
- separating solid cellular material from the remainder of the homogenate by centrifugation;
- solubilizing the solid cellular material by suspending it in an aqueous solution of sodium dodecyl sulfate (SDS) at a protein to SDS ratio of about 1:3;

extracting the β -HIFN from the aqueous phase with 2-butanol or 2-methyl-2-butanol or mixtures thereof by continuous cocurrent extraction;
 heating the 2-butanol or 2-methyl-2-butanol phase to about 60 °C for about 10 to 20 minutes; or
 aging the 2-butanol phase overnight;
 5 centrifuging the mixture and discarding the precipitated impurities;
 contacting the 2-butanol or 2-methyl-2-butanol phase with an aqueous buffer and adjusting the pH of the mixture to about 5.5 to precipitate the β -HIFN;
 collecting the precipitated β -HIFN by centrifugation;
 solubilizing the β -HIFN with distilled water or with an aqueous solution of sodium dodecyl sulfate at a
 10 protein to SDS ratio of about 1:3;
 adjusting the pH of the solution to about 9.5 and reducing the solubilized β -HIFN with dithiothreitol;
 purifying the reduced β -HIFN by chromatography;
 collecting the eluted fraction of the purified β -HIFN;
 further purifying the β -HIFN by gel chromatography;
 15 collecting the eluate containing the purified β -HIFN;
 adding sodium laurate to the eluate;
 adjusting the pH of the eluate to about 9-9.5;
 mixing the formulation;
 adjusting the pH of the formulation down to pH 7.0 to 8.0;
 20 and lyophilizing the β -HIFN sample, if desired.
 Ten mM dithiothreitol may be optionally included in the initial solubilization step and the mixture heated to about 90 °C for about 10 minutes.

The microorganisms transformed with β -HIFN encoding genes are grown in a suitable growth medium, typically to an optical density (OD) of at least about 10 at 680 nm, and preferably between about 50 and
 25 100 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The aqueous growth medium contains compounds that fulfill the nutritional requirements of the microorganism of choice. Growth media will typically contain assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (Review of Medical Microbiology, Lange Medical Publications, 14th Ed pp. 80-85 (1980)).
 30 Growth media for *E. coli* are well known in the art. Depending upon the particular solubilizing agent used in the invention process it may be desirable to minimize the amount of substances in the growth medium that may decrease the solubility of the solubilizing agent in water. For instance, potassium ions affect the solubility of SDS and, therefore, should be kept to a minimum when SDS is used in a solubilizing agent in the process, or removed by diafiltration following the concentration step.

35 Once the culture has reached the desired cell density, the cells are optionally killed or inactivated by heating or adding to the medium a cytotoxic agent such as chloroform or toluene that may be removed easily after the cells have been killed. The cells are thereafter optionally concentrated to about 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm) by crossflow filtration, centrifugation, or other conventional methods.

40 Following the concentration step, the cell membranes of the microorganisms are disrupted to facilitate the solubilization of the particulate matter in the concentrate. β -HIFN assays for biological activity indicate that much of the interferon is associated with (i.e., contained in or bound to) the cell membrane. Accordingly, disruption of the cell membrane enhances the contact of the solubilizing agent with the membranes and thus increases the rate at which the interferon associated with the membrane goes into
 45 solution. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling may be used in this step of the process. A preferred method is using a bead mill or a pressure homogenizer. Either before or after the disruption, the pH of the liquid phase of the concentrate or disruptate, as the case may be, is adjusted, if necessary, to a level that facilitates dissolution of the solubilizing agent and the particulate matter in the concentrate/disruptate. The pH may be so adjusted by
 50 adding suitable buffers or NaOH. In most instances a pH in the range of 7 to 8 is preferred.

After the cells have been disrupted the particulate matter can be separated from the liquid phase of the disruptate and resuspended in an aqueous medium buffered to the optimal pH for solubilization. The protein concentration of the cell suspension after solubilization is in the range of 2 to 15 mg/ml, preferably 6 to 8 mg/ml.

55 The solubilization of the particulate cellular material, including the β -HIFN, can be carried out concurrently with the disruption or sequentially following the disruption. It is preferably carried out as a separate step following the disruption. The solubilization is preferably carried to completion -- that is, substantially all of the particulate matter (e.g., protein, lipids, nucleic acids, phospholipids) in the disruptate is dissolved into

the aqueous medium. Substantially complete dissolution of the particulate matter is achieved by adding an appropriate solubilizing agent to the aqueous suspension. Surfactants (detergents) which have a suitable hydrophobic-hydrophilic balance to solubilize β -HIFN and which form a complex with β -HIFN that can be extracted into the organic phase can be used in the invention. Strong natural or synthetic anionic surfactants such as alkali metal salts of fatty acids and alkali metal alkyl sulfates may be used. Such agents will usually contain 10 to 14 carbon atoms. Sodium dodecyl sulfate (SDS) and sodium laurate are particularly preferred solubilizing agents. Examples of other solubilizing agents that can be used in the process include but are not limited to sodium dodecyl sulfonate, sodium decyl sulfate, sodium tetradecyl sulfate, sodium tridecyl sulfonate, sodium myristate, sodium caproylate, sodium dodecyl N-sarcosinate, and sodium tetradecyl N-sarcosinate.

The amount of solubilizing agent used in the solubilization depends upon the particular agent and the amount of protein to be solubilized. In most instances, solubilizing agent to protein weight ratios in the range of about 1:1 to 10:1 are sufficient. When SDS is used, an SDS to protein ratio of 1:1 to 5:1, preferably about 3:1, is used. Temperatures in the range of 15°C to 60°C are normally used in the solubilization. Mixing may be employed to enhance contact between the solution and particulate matter and thus decrease the time it takes to dissolve the cellular matter. The solubilization is considered complete when the solution is substantially clear. ODs of about 4.0 to 8.0 at 280 nm are characteristic of the end point of the solubilization process.

Following the solubilization, the ionic strength of the solution is adjusted, if necessary, to a level at which the solution and organic extractant are substantially immiscible. The ionic strength is in the range of about 0.05 to 0.15. Inorganic salts, including NaCl and the like, are added to the solution for this purpose. Such ionic strengths enable phase separation after the extraction. The extractants used in the process are alcohols such as 2-butanol, 2-methyl-2-butanol, or mixtures thereof. The mixtures preferably contain less than about 50% by volume of 2-methyl-2-butanol. 2-Butanol is the preferred extractant. The ability of these alcohols to extract human β -IFN from the solubilize is specific. The extractant is normally combined with the aqueous solution of β -HIFN in volume ratios in the range of 0.8:1 to 3:1, preferably about 1:1 (extractant:aqueous solution). The extraction can be carried out using conventional batch or continuous liquid-liquid extraction techniques and equipment. The extraction is normally carried out at about 20°C to 100°C and involves contact times in the range of about one minute to one hour. The optimum contact time depends upon the particular solubilizing agent and extractant combination. When SDS is used, shorter times in the above range can be used. When sodium laurate is used, longer times in the range must be used. The pH of the extraction mixture ranges between about 6 and 9, with a pH of about 7.5 being preferred when SDS is used and a pH of about 8.5 when sodium laurate is used.

Upon completion of the extraction, the aqueous phase and extractant phase are separated and the β -HIFN is isolated from the extractant phase. The particular isolation procedure used depends upon the solubilizing agent involved and the desired degree of purity of the final product. Various isolation techniques such as precipitation, molecular sieve chromatography, affinity chromatography, and electrophoresis are employed. In instances in which SDS is used, the β -HIFN together with other proteins are precipitated from the extractant by mixing the extractant solution with aqueous buffer at volume ratios of 2:1 to 5:1, preferably about 3:1, and reducing the pH, typically to the range of about 5 to 7. The recovery of β -HIFN in the pH range of 4 to 8 shows a downward trend in the recovery of the protein with increasing pH, with an appreciable loss in the recovery of greater than 60% at a pH of about 8. Separation of the precipitate from the supernatant and evaporation of residual extractant from the precipitate provide a product that is greater than about 90% pure protein provided that the pH of the precipitation step is greater than 5.5. This product also contains minor amounts of nucleic acids (<1% to 2% by weight) and SDS (<1% w/v). After further purification by methods known in the art, including but not limited to chromatography, sodium laurate is added at a pH of about 9 to 9.5 as described above for IL-2, and then the pH is adjusted to about 7.0 to 8.0 as described above. Prior to addition of sodium laurate the β -HIFN may be optionally reduced with appropriate reducing agents. Mercaptoethanol, glutathione, cysteine and dithiothreitol (DTT) are normally employed, DTT being the most preferred.

The β -HIFN thus isolated can be lyophilized or stored in solution pending use. Nontoxic, non-therapeutic, nonimmunogenic stabilizers may be optionally added to the β -HIFN. Diluents that can be used in the solutions for therapeutic or clinical administrations are selected from aqueous based vehicles commonly used to formulate pharmaceuticals for animal or human administration. The diluent should, of course, not affect the biological activity of the β -HIFN. Examples of such diluents include distilled water, physiological saline, Ringer's solution, dextrose solution, and Hank's solution. The same diluents can be used to reconstitute lyophilized β -HIFN.

The β -HIFN formulation may be used for anti-viral, anti-cancer, immunoregulatory and other therapeutic

purposes.

When used *in vivo* for therapy, the protein formulation is administered to the patient in therapeutically effective amounts (i.e., amounts that eliminate or reduce the patient's diseased condition). They will normally be administered parenterally, preferably intravenously. The dose and dosage regimen will depend upon the nature of the disease being treated and its population, the characteristics of the particular formulation, e.g., its protein concentration, the patient, and the patient's history. The amount of formulation administered will typically be in the range of 0.1 to 10 mg/kg of patient weight.

The invention process is further described by the following examples.

In the examples all parts and percentages are given by weight per volume and all temperatures are given by degrees Centigrade unless otherwise noted.

EXAMPLE 1

A mutein IL-2 designated des-Ala Ser₁₂₅ IL-2 was recovered from *E. coli*. the amino acid sequence of this IL-2 is different from that of the native molecule in that the cysteine at position 125 has been changed to serine and the initial N-terminal alanine residue is missing. The strain of des-Ala Ser₁₂₅ IL-2-producing *E. coli* used in this example was deposited at the American Type Culture Collection on March 6, 1984 under accession number 39,626.

The *E. coli* thus transformed were grown in a fermenter using the following growth medium.

20	(NH ₄) ₂ SO ₄	150 mM
	KH ₂ PO ₄	21.6 mM
25	Na ₃ Citrate	1.5 mM
	ZnSO ₄ · 7H ₂ O	30 mM
	MnSO ₄ · H ₂ O	30 mM
	CuSO ₄ · 5H ₂ O	1 mM
30	pH adjusted to 6.50 with 2.5 N NaOH autoclaved	
	Sterile Additions (post autoclave)	
35	MgSO ₄ · 7H ₂ O	3 mM
	FeSO ₄	100 μM
	L-tryptophan	14 mg/l
40	Thiamine-HCl	20 mg/l
	Glucose	5 g/l
	Tetracycline	5 mg/l
45	Ethanol (optional)	2%
	Casamino acids	2%

Dow Corning Antifoam B, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand. The pH of the fermenter was maintained at 6.8 with 5 N KOH. Residual glucose was maintained between 5 and 10 g/l, dissolved oxygen at 40%, and temperature at 37 ± 1 °C. The casamino acids (20% stock solution) were added when the OD₆₈₀ was about 10. Harvest was made three hours after the OD₆₈₀ reached about 20.

The harvested material was concentrated by hollow fiber filtration and/or centrifugation. Twenty to forty g (wet weight) of the concentrate were resuspended in 200 ml of 50 mM Tris·HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.1-8.5) (Tris/EDTA buffer). The suspension was centrifuged at 3,000-4,000 × g for 10 minutes, the supernatant was removed, and the solids were resuspended in 200 ml Tris/EDTA buffer at 4 °C. The suspension was loaded into a sonicator (Heat Systems, Model W-375) and

sonicated at 4 °C for 45 minutes (end point = OD₆₈₀ reduction of about 85%) using large probe, pulsing with 50% duty on power setting "9". An alternative disruption technique is to pass the suspension three times through a Manton-Gaulin homogenizer. Cellular debris was separated from the disruptate by centrifuging at 4,500 x g for 10 minutes.

5 The cellular debris was suspended in 50 mM Tris·HCl, 1 mM EDTA pH 8.5 buffer at room temperature at a ratio of about 1:4.5 (w/v). Dithiothreitol (DTT) was added to a final concentration of 25 mM. 8 M urea in the same Tris/EDTA buffer was slowly added to the suspension over 5 minutes with rapid stirring (final urea concentration, 4 M). After continued slow stirring at room temperature for 30 minutes, the suspension was centrifuged at 12,000 x g for 15 minutes to recover extracted cellular debris. (If a solid phase does not form,
10 the supernatant is withdrawn, and equal volume of Tris/EDTA buffer is added and the mixture is centrifuged.)

The extracted cellular debris was then resuspended in 9 ml of 50 mM sodium phosphate, 1 mM EDTA at pH 7.0. The suspension was then solubilized by adding solid SDS to the suspension to a final concentration of 5% w/v.

15 The 5% SDS solution was diluted to 2% SDS with 0.1 M Na₂HPO₄, pH 8.0. The protein concentration was determined, the pH was adjusted to 8.5, and DTT to 50 mM and EDTA to 2 mM were added. The mixture was heated to 40 °C under N₂ to reduce the IL-2. The mixture was then cooled and the pH was adjusted to 5.0.

The solution was then extracted at a 1:1 ratio (v/v) with 2-butanol containing 1 mM DTT at room
20 temperature.. Residence time was 2-2.5 minutes. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic extract was separated and its pH was adjusted to 8.0 with NaOH. The extract was then added slowly to 0.1% SDS in 10 mM Na₂HPO₄, 2 mM DTT, pH 6 and stirred for 15-20 minutes. The resulting precipitate was separated and the resulting paste was resuspended in 5% SDS in PBS.

25 The solution was clarified by centrifugation and then 200 ml of the clarified solution was reduced using 10 mM DTT in the presence of 2.5 mM EDTA at pH 8.0 and 60 °C for 30 minutes. The suspension was centrifuged at 35K for 2 hours. The supernatant (35 ml) was loaded on a S-200 (K-50., Pharmacia) column and eluted with acetate pH 5.5 (50 mM), DTT (2 mM), EDTA (1 mM) and SDS (0.1%) at a rate of 1.5 ml/min. The S-200 pool (270 ml, A₂₈₀ = 1.77) was about 33% pure as determined by HPLC.

30 A portion of the S-200 pool (35 ml) was acidified with trifluoroacetic acid (TFA) to pH 2.0, and then loaded at 2.5 ml/min. on a semi-preparative (1.3 cm) C-4 Vydac column that was freshly prepared. This was done three times with 35 ml each loading. The solvent used for this semi-preparative purification was acetonitrile (0.1% TFA, buffer B) and the gradient that was used was 0% to 45% buffer B in 15 minutes followed by 45% to 75% of B in 200 minutes. The IL-2 pool came out as 76 ml (three runs) with an
35 A₂₈₀ = 0.326 that corresponds to about 25 mg of IL-2 and which is about 15% yield. This HPLC run was diluted into 1600 ml of Na₂HPO₄ buffer (0.1 M, pH 7.0, 0.1% SDS) and then concentrated to 50 ml by using an Amicon cell equipped with a 76 mm PM-10 membrane. The concentrate was washed with three volumes of 50 ml each of Na₂HPO₄ (50 mM) pH 7 buffer which contained 0.1% SDS. The final volume was 43 ml with an A₂₈₀ = 0.65.

40 Before the oxidation was carried out, the total thiol content of the protein solution was determined with 2,2'-dithiodipyridine. This determination was necessary in order to calculate the minimum theoretical amount of o-iodosobenzoic acid that had to be added to the IL-2 solution to achieve complete oxidation. o-iodosobenzoic acid solution (1 mM, 50 ml) was prepared by dissolving the compound (13.4 mg) in about 45 ml of H₂O by sonicating the mixture for a few minutes and then by stirring and slowly adding NaOH (1 N) to
45 dissolve the acid. The alkaline solution was added to obtain a final pH of 8.0 to 8.5. The volume of the oxidant solution was adjusted to a final volume of 50 ml. A sulfhydryl group determination was done in order to determine the total amount of oxidant needed for a complete oxidation. This corresponds to the total thiol concentration divided by two plus a 15 micromolar excess of the oxidant. The controlled oxidation was performed by adding the o-iodosobenzoic acid solution at a flow rate of 0.5 ml/hour to the IL-2 solution (50
50 mM Na₂HPO₄, pH 7 or 7.5). The degree of the oxidation was monitored by reverse phase HPLC. The oxidation was stopped by lowering the pH of the solution to 5.5 using concentrated acetic acid. HPLC analysis of the oxidized product showed that it comprised about 80% of the desired oxidized IL-2, about 13% undesired isomers (the isomers were collected, assayed for IL-2 activity and found to be inactive) and about 6% reduced (unoxidized) IL-2.

55 The oxidized product was separated from iodosobenzoate and iodobenzoate by G-25 column chromatography using 0.1% SDS, 50 mM sodium acetate, 1 mM EDTA, pH 5.5. The IL-2 was then separated from the solution by RP-HPLC as follows. The solution was diluted 10-fold in 0.1% trifluoroacetic acid (TFA) and was applied to a 4.6 mm I.D. x 5 cm L. Brownlee Aquapore RP-300 column equilibrated in

0.1% TFA. The IL-2 was eluted with a gradient of 30-60% acetonitrile containing 0.1% TFA over 45 minutes. The resulting purified recombinant IL-2 product had an IL-2 content greater than about 95% as determined by reducing SDS-PAGE analysis and an endotoxin content of less than about 0.1 nanograms/mg of IL-2, and it was substantially free of pyrogens as determined by the U.S. P. rabbit pyrogen test at a dosage of 3.3×10^5 U/kg.

The process up to this point is shown in Figure 1. A variation of this process may be used to produce IL-2 on a larger scale. Thus, there may be (1) minor changes in the buffers, (2) use of an acetic acid-propanol solvent system in the RP-HPLC, and (3) the inclusion of post-oxidation dilution/diafiltration, S-200 gel filtration, and ultrafiltration steps. The process up to HPLC as shown in Figure 1 may be modified with various refinements, for example, following the second S-200 column pass, in 1% SDS, the IL-2 solution may be diluted 1:10 to give a 0.1% SDS concentration and then diafiltered against 10 mM phosphate buffer at a pH of 7.5 and 5 ppm SDS. The solution may be then concentrated as required for appropriate use dosage.

A total of 100 ml of the HPLC pool described above containing 250 mg oxidized, purified IL-2 in 0.1% trifluoroacetic acid and 60% acetonitrile was precipitated by adding 6 ml of 0.5 M Na_2HPO_4 to the pool. The precipitate was then collected by centrifuging at $5,000 \times g$ for 15 minutes. The solid centrifugate was then redissolved in 120 ml of 0.5% sodium laurate and 10 mM Na_2HPO_4 . This solution was loaded on a Sephadex G-25 column, 5 x 80 cm using a column buffer of 10 mM Na_2HPO_4 , pH 9.1 and a flow rate of 20 ml/min. The IL-2 was then collected from the column in a pool of 150 ml containing 1.5 mg IL-2 per ml. The concentration of sodium laurate in this pool was less than or equal to 0.01% and the SDS concentration, as determined by the acridine orange assay method for SDS described by *Anal Biochem*, 118, 138-141 (1981), was less than or equal to 0.4 $\mu\text{g}/\text{mg}$ IL-2.

The IL-2 collected from the column was combined with water, sodium laurate, Na_2HPO_4 and mannitol to a pH of 9.1 to yield final concentrations of:

IL-2	0.25 mg/ml
Sodium laurate	0.07%
Na_2HPO_4	10 mM
Mannitol	5%

The final concentration of SDS as determined by the acridine orange assay described above was less than or equal to 0.4 $\mu\text{g}/\text{mg}$ IL-2 or 0.1 $\mu\text{g}/\text{ml}$.

The formulation at pH 9.1 was adjusted to pH 7.5 by adding 1 M HCl thereto. It was then lyophilized by known techniques.

The lyophilized formulation was virtually free of SDS, yet the IL-2 was still soluble in the formulation.

EXAMPLE 2

This example illustrates the effect of final pH on solubility of the IL-2. The procedure of Example 1 was followed except that the formulation at pH 9.1 had final concentrations of:

IL-2	0.25 mg/ml
Sodium laurate	0.03%
Na_2HPO_4	10 mM
Mannitol	2.5%

The final concentration of IL-2 was 0.64 mg/ml. This formulation was split into two portions, one which was adjusted to pH 7.5 with 1 N HCl to yield a clear (IL-2 soluble) solution and one adjusted to pH 7.0 to yield a solution which is slightly hazy, indicating some IL-2 insolubility. The formulation when adjusted to pH 6.5 became hazier and was very hazy after 1 minute. When sodium hydroxide was added to the formulation to a pH of 7.5, the solution became almost completely clear.

EXAMPLE 3

This example illustrates the effect of sodium laurate concentration on IL-2 solubility. The procedure of Example 2 was followed except that 5% mannitol was employed rather than 2.5% and no sodium laurate was added to the formulation before adjustment to pH 7.5. The amounts of ingredients were:

- 9.75 ml of the IL-2 pool
- 0.5 ml 0.5 M Na_2HPO_4
- 5.0 ml 25% mannitol
- 11.0 ml H_2O

The formulation after adjustment to pH 7.5 turned hazy, indicating that the IL-2 was not soluble therein.

A formulation containing 0.01% sodium laurate was prepared using the following concentrations of ingredients:

- 9.75 ml of the IL-2 pool
- 0.5 ml 0.5 M Na_2HPO_4
- 2.5 ml mannitol (25%)
- 0.25 ml 1% sodium laurate
- 10 ml H_2O

The formulation after adjustment to pH 7.5 was clear, indicating that the IL-2 was soluble in the mixture.

A formulation containing 0.1% sodium laurate was prepared using the following concentrations of ingredients:

- 9.75 ml of the IL-2 pool
- 0.5 ml 0.5 M Na_2HPO_4
- 2.5 ml mannitol (25%)
- 2.5 ml 1% sodium laurate
- 10 ml H_2O

The formulation after adjustment to pH 7.5 was clear, indicating that the IL-2 was soluble in the mixture.

A formulation comprising 0.06% sodium laurate was prepared using the following concentrations of ingredients:

- 9.75 ml of the IL-2 of this example
- 0.5 ml Na_2HPO_4
- 2.5 ml 25% mannitol
- 1.5 ml 1% sodium laurate
- 8 ml H_2O

When the pH of this formulation was adjusted to 7.5 with 1 N HCl the solution remained clear.

A control formulation containing 1% SDS rather than sodium laurate at pH 7 was prepared using the following concentrations of ingredients:

- 9.75 ml of the IL-2 pool
- 2.5 ml 10% SDS
- 0.5 ml 0.5 M Na_2HPO_4 , pH 7
- 2.5 ml 25% mannitol
- 9.75 ml H_2O

The formulation was found to be clear.

Ultraviolet scans were performed on the formulations with 0%, 0.01%, 0.03% and 0.1% sodium laurate and with 1% SDS. These scans indicated that the 0.1% laurate formulation has the same stability as the 1% SDS formulation.

A control formulation containing 0.1% sodium octanoate was prepared using the following concentrations of ingredients:

- 9.75 ml of the IL-2 pool
- 0.5 ml 0.5 M Na_2HPO_4
- 2.5 ml 25% mannitol
- 0.25 ml 1% sodium octanoate
- 10ml H_2O

When the formulation was adjusted to pH 7.5 it appeared slightly hazy. when the sodium octanoate was added to a level of 0.2%, the formulation was still hazy. Therefore, sodium octanoate (a C_8 fatty acid salt) is not effective as a stabilizer as is sodium laurate (a C_{12} fatty acid salt).

When the above experiment was repeated using sodium tetradecanoate (a C_{14} fatty acid sodium salt), the salt would not remain in solution. When the above experiment was repeated using sodium decanoate (a C_{10} fatty acid salt), the formulation was hazy. Only the C_{12} fatty acid salt was effective among these fatty

acids tested.

EXAMPLE 4

5 The solvents for RP-HPLC were 6% acetic acid/water and 6% acetic acid/2-propanol in the procedure of Example 1. The IL-2 elutes at about 40% 2-propanol during gradient elution. The HPLC pool was diluted with one volume of 0.8 N sodium hydroxide. The IL-2 precipitated and was collected by centrifugation. The precipitated IL-2 was then redissolved in sodium laurate and Na₂HPO₄ and formulated as described in Example 1.

10 It is expected that parenteral injection of the formulations containing the recombinant IL-2 or β -HIFN protein and sodium laurate in effective amounts into patients requiring treatment with the protein will be effective because the protein is soluble at physiological pH and the exposure of the protein to strongly alkaline pH is eliminated.

The present invention is seen to provide a pharmaceutical composition suitable for parenteral injection 15 into patients which contains recombinant IL-2 or β -HIFN and an effective amount of sodium laurate. The sodium laurate not only has the properties necessary to stabilize the protein but also can be readily metabolized once in the blood so that it would not be, for example, concentrated in the liver. Total fatty acids, including sodium laurate (sodium dodecanoate), are present in the blood at levels of 2-4 mg/ml or about 12 to 20 g total fatty acids in the whole blood system. The injected sodium laurate will bind quickly to 20 serum albumin and enter the metabolic cycle of the bulk fatty acids, with no problem of hemolysis or sequestration of the sodium laurate.

In addition, the process herein represents a method of formulating IL-2 or IFN- β protein containing less than 0.4 μ g/ml of SDS, as opposed to current levels of about 5-10 μ g/ml of SDS in IFN formulations and about 150 μ g/ml of SDS in IL-2 formulations. In addition, the proteins obtained by this process are 25 substantially free of other materials in the cell membrane disruptate. The process herein results in recovery of the proteins in relatively highly pure form with trace or no amounts of SDS, which proteins can be reconstituted into therapeutically acceptable formulations.

Claims

- 30 1. A stable composition suitable for parenteral injection into animals or humans and containing a therapeutically effective amount of a recombinant interleukin-2 or interferon- β protein purified to contain less than 4 μ g sodium dodecyl sulfate per mg protein dissolved in an inert carrier medium comprising sodium laurate at a pH of 7.0 to 8.0.
- 35 2. A composition according to claim 1 wherein the protein is a mutein in which a cysteine residue not essential to biological activity has been deleted or replaced with another amino acid if the protein is interferon- β or replaced with another amino acid if the protein is interleukin-2.
- 40 3. A composition according to claim 1 or 2 wherein the protein is des-ala₁ser₁₂₅ IL-2 or Ser₁₇IFN- β , the medium is aqueous, and the pH is 7.5 to 7.7.
4. A composition according to any one of claims 1-3 and which has less than 0.4 μ g/ml of sodium dodecyl sulfate and further contains mannitol.
- 45 5. The use of sodium laurate in stabilizing an interleukin-2 or interferon- β protein so as to render said protein suitable for administration to animals or humans.
- 50 6. A process for formulating recombinant interleukin-2 or interferon- β protein in an inert carrier medium suitable for parenteral administration to animals or humans comprising (a) extracting the protein from a disruptate of a host organism transformed to produce the protein, (b) purifying the extracted protein to contain less than 4 μ g sodium dodecyl sulfate per mg protein, (c) mixing the purified protein with said medium at a pH of about 9 to 9.5 in the presence of sodium laurate, and (d) adjusting the pH of said mixture to between 7.0 and 8.0.
- 55 7. A process according to claim 6 wherein the disruptate of step (a) is of a cell membrane of a microorganism host, the extracting is conducted using sodium dodecyl sulfate, and the concentration of sodium laurate is between 0.01 and 0.1% by weight per volume, depending on the pH of the solution.

8. A process for formulating recombinant interleukin-2 or Interferon- β protein suitable for parenteral injection into animals and humans comprising:
- (a) disrupting the cell membrane of a transformed microorganism containing the protein;
 - (b) extracting the disruptate with an aqueous solution of a chaotropic agent that extracts the undesired proteins selectively from the cellular material;
 - (c) solubilizing the protein in the solid phase of the extraction mixture with an aqueous solution of a solubilizing agent that forms a water soluble complex with the protein, said solution containing a reducing agent;
 - (d) separating the protein from the resulting solution in the presence of the reducing agent;
 - (e) oxidizing the product of step (d);
 - (f) purifying the resulting oxidized product by reverse-phase high performance liquid chromatography;
 - (g) precipitating the pool from step (f) and collecting the precipitate;
 - (h) redissolving the precipitate in the presence of a chaotropic agent;
 - (i) passing the redissolved precipitate through a gel filtration column and collecting the protein from the column;
 - (j) combining the protein from step (i) with an inert carrier medium at a pH between 9 and 9.5 comprising sodium laurate;
 - (k) adjusting the pH of the combination of step (j) to between 7.0 and 8.0, inclusive, to obtain a solution; and
 - (l) lyophilizing the solution of step (k) to obtain the final formulation.
9. A process according to claim 8 wherein the chaotropic agent used in step (h) is selected from the group consisting of sodium laurate, urea, guanidine hydrochloride, and sodium thiocyanate, and the solvent system used for the chromatography in step (f) is trifluoroacetic acid and acetonitrile.
10. A process for formulating des-ala₁ser₁₂₅ IL-2 for parenteral administration to humans comprising:
- (a) disrupting the cell membrane of transformed *E. coli* containing the IL-2;
 - (b) extracting the disruptate with an aqueous solution of urea at a basic pH;
 - (c) solubilizing the IL-2 in the solid phase of the extraction mixture with an aqueous solution of sodium dodecyl sulfate, said solution containing a reducing agent;
 - (d) separating the IL-2 from the resulting solution in the presence of the reducing agent;
 - (e) oxidizing the product of step (d);
 - (f) purifying the resulting oxidized product by reverse-phase high performance liquid chromatography using a mixture of 0.1% trifluoroacetic acid and acetonitrile;
 - (g) precipitating the pool from step (f) using Na₂HPO₄ and collecting the precipitate;
 - (h) redissolving the precipitate in sodium laurate containing Na₂HPO₄;
 - (i) passing the redissolved precipitate through a desalting column and collecting the IL-2 from the column;
 - (j) combining the IL-2 with 0.1% by weight per volume sodium laurate with Na₂HPO₄ at pH 9.0-9.5;
 - (k) adjusting the pH of the combination from step (j) to between 7.0 and 8.0, inclusive, where if the pH is adjusted to less than 8.0 the combination is filtered to remove excess sodium laurate; and
 - (l) lyophilizing the combination from step (k) to obtain the final formulation.
11. A process for preparing a pharmaceutical composition comprising formulating an interleukin-2 or interferon- β protein to provide said protein purified to contain less than 4 μ g sodium dodecyl sulfate per mg protein dissolved in an inert carrier medium comprising sodium laurate at a pH of 7.0 to 8.0.

Revendications

1. Composition stable convenant à l'injection parentérale à des animaux ou à l'homme et contenant une quantité thérapeutiquement efficace d'une protéine recombinée interleukine-2 ou interféron- β , purifiée pour contenir moins de 4 μ g de dodécylsulfate de sodium par mg de protéine, dissoute dans un milieu véhicule inerte comprenant du laurate de sodium à un pH de 7,0 à 8,0.
2. Composition selon la revendication 1, dans laquelle la protéine est une mutéine dans laquelle un reste de cystéine, qui n'est pas essentiel à l'activité biologique, a été supprimé ou remplacé par un autre amino-acide si la protéine est l'interféron- β , ou remplacé par un autre amino-acide si la protéine est

l'interleukine-2.

3. Composition selon la revendication 1 ou 2, dans laquelle la protéine est dés-ala₁ ser₁₂₅ IL-2 ou Ser₁₇ IFN- β , le milieu est aqueux et le pH est de 7,5 à 7,7.
- 5 4. Composition selon l'une quelconque des revendications 1 à 3 et qui a moins de 0,4 μ g/ml de dodécylsulfate de sodium et de plus contient du mannitol.
- 10 5. Utilisation du laurate de sodium dans la stabilisation d'une protéine interleukine-2 ou interféron- β afin de rendre ladite protéine appropriée à l'administration aux animaux ou à l'homme.
- 15 6. Procédé pour la formulation d'une protéine recombinée interleukine-2 ou interféron- β dans un milieu véhicule inerte approprié à l'administration parentérale aux animaux ou à l'homme, comprenant (a) l'extraction de la protéine à partir d'un produit de dissociation d'un organisme hôte transformé pour produire la protéine, (b) la purification de la protéine extraite pour qu'elle contienne moins de 4 μ g de dodécylsulfate de sodium par mg de protéine, (c) le mélange de la protéine purifiée avec ledit milieu à un pH d'environ 9 à 9,5 en présence de laurate de sodium et (d) l'ajustement du pH dudit milieu entre 7,0 et 8,0.
- 20 7. Procédé selon la revendication 6, dans lequel le produit de dissociation de l'étape (a) est celui d'une membrane cellulaire d'un microorganisme hôte, l'extraction est effectuée avec du dodécylsulfate de sodium et la concentration du laurate de sodium est entre 0,01 et 0,1 % en poids par volume, selon le pH de la solution.
- 25 8. Procédé pour la formulation d'une protéine recombinée interleukine-2 ou interféron- β appropriée à l'injection parentérale à des animaux et à l'homme, comprenant :
 - (a) la dissociation de la membrane cellulaire d'un microorganisme transformé contenant la protéine ;
 - (b) l'extraction du produit de dissociation avec une solution aqueuse d'un agent chaotrope qui extrait sélectivement les protéines indésirables du matériau cellulaire ;
 - 30 (c) la solubilisation de la protéine dans la phase solide du mélange d'extraction avec une solution aqueuse d'un agent solubilisant qui forme un complexe soluble dans l'eau avec la protéine, ladite solution contenant un agent réducteur ;
 - (d) la séparation de la protéine d'avec la solution obtenue en présence de l'agent réducteur ;
 - (e) l'oxydation du produit de l'étape (d) ;
 - 35 (f) la purification du produit oxydé obtenu par chromatographie liquide haute performance en phase inverse ;
 - (g) la précipitation de l'ensemble de l'étape (f) et la récolte du précipité ;
 - (h) la redissolution du précipité en présence d'un agent chaotrope ;
 - (i) le passage du précipité redissous à travers une colonne de filtration sur gel et la récolte de la protéine de la colonne ;
 - 40 (j) la combinaison de la protéine de l'étape (i) avec le milieu véhicule inerte à un pH entre 9 et 9,5 comprenant du laurate de sodium ;
 - (k) l'ajustement du pH de la combinaison de l'étape (j) entre 7,0 et 8,0 inclusivement, pour obtenir une solution ; et (l) la lyophilisation de la solution de l'étape (k) pour obtenir la formulation finale.
- 45 9. Procédé selon la revendication 8, dans lequel l'agent chaotrope utilisé dans l'étape (h) est choisi dans le groupe constitué par le laurate de sodium, l'urée, le chlorhydrate de guanidine et le thiocyanate de sodium et le système solvant utilisé pour la chromatographie dans l'étape (f) est constitué d'acide trifluoroacétique et d'acétonitrile.
- 50 10. Procédé pour la formulation de dés-ala₁ ser₁₂₅ IL-2 pour l'administration parentérale à l'homme, comprenant :
 - (a) la dissociation de la membrane cellulaire d'un *E. coli* transformé contenant l'IL-2 ;
 - (b) l'extraction du produit de dissociation avec une solution aqueuse d'urée à un pH basique ;
 - 55 (c) la solubilisation de l'IL-2 dans la phase solide du mélange d'extraction avec une solution aqueuse de dodécylsulfate de sodium, ladite solution contenant un agent réducteur ;
 - (d) la séparation de l'IL-2 d'avec la solution obtenue en présence de l'agent réducteur ;
 - (e) l'oxydation du produit de l'étape (d) ;

- (f) la purification du produit oxydé obtenu par chromatographie liquide haute performance en phase inverse en utilisant un mélange de 0,1 % d'acide trifluoroacétique et d'acétonitrile ;
 (g) la précipitation de l'ensemble de l'étape (f) en utilisant du Na_2HPO_4 et la récolte du précipité ;
 (h) la redissolution du précipité dans du laurate de sodium contenant du Na_2HPO_4 ;
 5 (i) le passage du précipité redissous à travers une colonne de dessalement et la récolte de l'IL-2 à partir de la colonne ;
 (j) la combinaison de l'IL-2 avec 0,1 % en poids par volume de laurate de sodium avec du Na_2HPO_4 à pH 9,0-9,5 ;
 (k) l'ajustement du pH de la combinaison de l'étape (j) entre 7,0 et 8,0 inclusivement et, si le pH est
 10 ajusté à moins de 8,0, la filtration de la combinaison pour éliminer l'excès de laurate de sodium ; et
 (l) la lyophilisation de la combinaison de l'étape (k) pour obtenir la formulation finale.

11. Procédé pour préparer une composition pharmaceutique comprenant la formulation d'une protéine interleukine-2 ou interféron- β , pour fournir ladite protéine purifiée afin qu'elle contienne moins de 4 μg
 15 de dodécylsulfate de sodium par mg de protéine, dissoute dans un milieu véhicule inerte comprenant du laurate de sodium à un pH de 7,0 à 8,0.

Patentansprüche

- 20 1. Stabile Zusammensetzung, geeignet für eine parenterale Injektion in Tiere oder Menschen, enthaltend eine therapeutisch wirksame Menge eines rekombinanten Interleukin-2- oder Interferon- β -Proteins, aufgereinigt um weniger als 4 μg Natriumdodecylsulfat pro mg Protein zu enthalten, gelöst in einem inerten, Natriumlaurat enthaltenden Trägermedium bei einem pH von 7,0 bis 8,0.
- 25 2. Zusammensetzung nach Anspruch 1, in der das Protein ein Mutein ist, in dem ein für die biologische Aktivität nicht essentieller Cysteinrest deletiert, oder durch eine andere Aminosäure ersetzt worden ist, wenn das Protein Interferon- β ist, oder durch eine andere Aminosäure ersetzt worden ist, wenn das Protein Interleukin-2 ist.
- 30 3. Zusammensetzung nach Anspruch 1 oder 2, in der das Protein des-Ala₁Ser₁₂₅ IL-2 oder Ser₁₇IFN- β ist, das Medium wasserhaltig und der pH 7,5 bis 7,7 ist.
4. Zusammensetzung nach einem der Ansprüche 1 bis 3, die weniger als 0,4 $\mu\text{g}/\text{ml}$ Natriumdodecylsulfat aufweist und ferner Mannit enthält.
- 35 5. Verwendung von Natriumlaurat zur Stabilisierung eines Interleukin-2- oder Interferon- β -Proteins, um das Protein für die Verabreichung an Tiere oder Menschen geeignet zu machen.
- 40 6. Verfahren zur Formulierung eines rekombinanten Interleukin-2- oder Interferon- β -Proteins in einem inerten Trägermedium, geeignet für eine parenterale Verabreichung an Tiere oder Menschen, umfassend
 (a) die Extraktion des Proteins aus einem Aufschluß eines zur Erzeugung des Proteins transformierten Wirtsorganismus,
 (b) die Reinigung des extrahierten Proteins, so daß es weniger als 4 μg an Natriumdodecylsulfat pro
 45 mg Protein enthält,
 (c) das Vermischen des gereinigten Proteins mit dem Medium bei einem pH von etwa 9 bis 9,5 in Gegenwart von Natriumlaurat und
 (d) das Einstellen des pH von dem Gemisch zwischen 7,0 und 8,0.
- 50 7. Verfahren nach Anspruch 6, in dem der Aufschluß von Schritt (a) der einer Zellmembran eines Wirtsmikroorganismus ist, die Extraktion mit Natriumdodecylsulfat durchgeführt wird, und die Konzentration von Natriumlaurat zwischen 0,01 und 0,1 Gew.-% pro Volumen, abhängig von dem pH der Lösung, ist.
- 55 8. Verfahren zur Formulierung eines rekombinanten Interleukin-2- oder Interferon- β -Proteins, geeignet für eine parenterale Injektion in Tiere und Menschen, umfassend:
 (a) das Aufschließen der Zellmembran eines transformierten, das Protein enthaltenden Mikroorganismus;

- (b) die Extraktion des Aufschlusses mit einer wäßrigen Lösung eines chaotropen Mittels, das die ungewünschten Proteine selektiv aus dem zellulären Material extrahiert;
- (c) die Solubilisierung des Proteins in der festen Phase des Extraktionsgemisches mit einer wäßrigen Lösung eines solubilisierenden Mittels, das einen wasserlöslichen Komplex mit dem Protein bildet, wobei die Lösung ein Reduktionsmittel enthält;
- (d) die Abtrennung des Proteins aus der erhaltenen Lösung in Gegenwart des Reduktionsmittels;
- (e) die Oxidation des Produktes von Schritt (d);
- (f) die Reinigung des erhaltenen, oxidierten Produktes durch Umkehrphase-Hochleistungsflüssigkeitschromatographie;
- (g) die Fällung der gesammelten Fraktionen von Schritt (f) und Sammeln des Niederschlags;
- (h) das Lösen des Niederschlags in Gegenwart eines chaotropen Mittels;
- (i) das Durchlaufen des wieder gelösten Niederschlags durch eine Gelfiltrationssäule und Sammeln des Proteins aus der Säule;
- (j) das Vereinigen des Proteins von Schritt (i) mit einem inerten, Natriumlaurat enthaltenden Trägermedium bei einem pH zwischen 9 und 9,5;
- (k) das Einstellen des pH der Zusammensetzung von Schritt (j) zwischen 7,0 und 8,0 zum Erhalt einer Lösung; und
- (l) die Lyophilisierung der Lösung von Schritt (k), um die endgültige Formulierung zu erhalten.
9. Verfahren nach Anspruch 8, in dem das in Schritt (h) verwendete chaotrope Mittel aus der Gruppe Natriumlaurat, Harnstoff, Guanidin-hydrochlorid und Natriumthiocyanat ausgewählt ist, und das für die Chromatographie in Schritt (f) verwendete Lösungsmittelsystem Trifluoressigsäure und Acetonitril ist.
10. Verfahren zur Formulierung von des-Ala₁Ser₁₂₅ IL-2 zur parenteralen Verabreichung an Menschen, umfassend:
- (a) den Aufschluß der Zellmembran von IL-2 enthaltenden, transformierten *E. coli*;
- (b) die Extraktion des Aufschlusses mit einer wäßrigen Lösung von Harnstoff bei einem basischen pH;
- (c) die Solubilisierung des IL-2 in der festen Phase des Extraktionsgemisches mit einer wäßrigen Lösung von Natriumdodecylsulfat, wobei die Lösung ein Reduktionsmittel enthält;
- (d) die Abtrennung des IL-2 aus der erhaltenen Lösung in Gegenwart eines Reduktionsmittels;
- (e) die Oxidierung des Produktes von Schritt (d);
- (f) die Reinigung des erhaltenen oxidierten Produktes durch Umkehrphase-Hochleistungsflüssigkeitschromatographie mit einem Gemisch von 0,1 % Trifluoressigsäure und Acetonitril;
- (g) die Fällung der gesammelten Fraktionen von Schritt (f) mit Na₂HPO₄ und Sammeln des Niederschlags;
- (h) die Lösung des Niederschlags in Na₂HPO₄ enthaltendem Natriumlaurat;
- (i) das Durchlaufen des gelösten Niederschlags durch eine Entsalzungssäule und Sammeln des IL-2 aus der Säule;
- (j) das Vereinigen des IL-2 mit 0,1 Gew.-% pro Volumen Natriumlaurat mit Na₂HPO₄ bei pH 9,0 bis 9,5;
- (k) die Einstellung des pH der Zusammensetzung von Schritt (j) zwischen 7,0 und 8,0, wobei die Zusammensetzung, wenn der pH auf weniger als 8,0 eingestellt ist, filtriert wird um überschüssiges Natriumlaurat zu entfernen; und
- (l) die Lyophilisierung der Zusammensetzung von Schritt (k), um die endgültige Formulierung zu erhalten.
11. Verfahren zur Herstellung eines Arzneimittels, umfassend die Formulierung eines Interleukin-2- oder Interferon-β-Proteins für die Bereitstellung des Proteins, das so aufgereinigt ist, daß es weniger als 4 µg Natriumdodecylsulfat pro mg Protein enthält, gelöst in einem inerten, Natriumlaurat enthaltenden Trägermedium bei einem pH von 7,0 bis 8,0.

FIG. 1

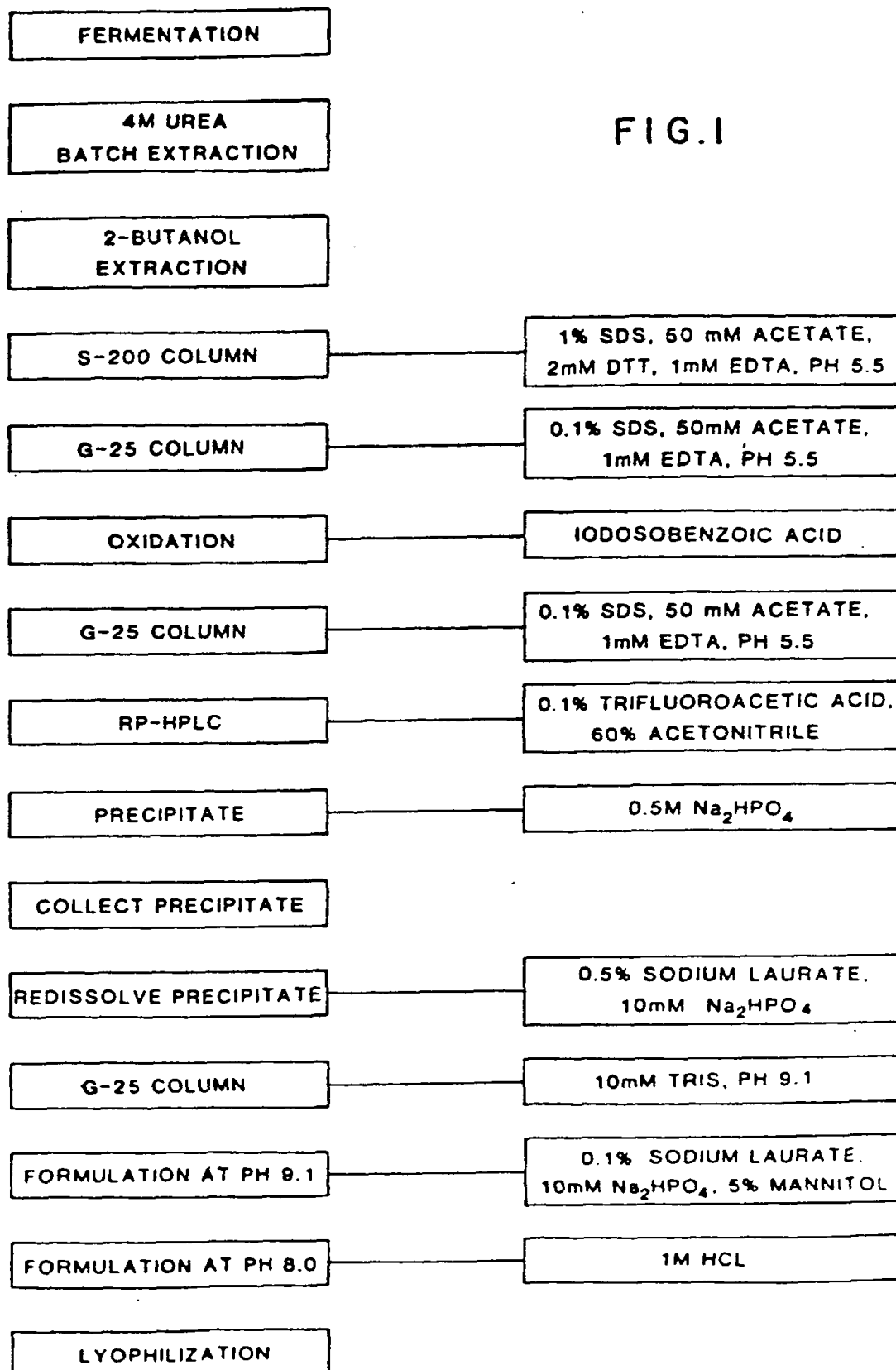


FIG.2

